Targeting RNA G-quadruplexes as new treatment strategy for *C9orf72* ALS/FTD

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The recent discovery of a pathogenic expansion of a $(GGGGCC)_n$ repeat in the *C9orf72* gene in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) led to a burst of mechanistic discoveries. In this issue, Simone *et al* (2018) describe novel compounds targeting the G-quadruplex (G-Q) structure of the $(GGGGCC)_n$ repeat RNA that alleviate the hallmarks of *C9orf72* disease in patient-derived neurons and increase survival in a *Drosophila* model. Lack of overt off-target effects and toxicity suggest that these small molecules are promising lead compounds to the development of a therapy.

See also: **R Simone *et al*** (January 2018)

S ince the discovery of the $(GGGGCC)_n$ repeat expansion upstream of the coding region of *C9orf72* as the most common genetic cause of ALS and FTD, tremendous progress toward understanding disease mechanisms and developing therapies has been made (Edbauer & Haass, 2016). The repeat RNA forms small foci within the nucleus and is thought to sequester several RNA-binding proteins and thereby alter gene expression and splicing. Surprisingly, both sense and antisense transcripts are translated in all reading frames by an unconventional mechanism into five co-aggregating dipeptide repeat (DPR) proteins: poly-GA, poly-GP, poly-GR, poly-PA, and poly-PR. The DPRs also bind key cellular proteins (including RNA-binding proteins), and their relative role in pathogenesis is under intense investigation. The so-called repeat-associated non-ATG (RAN) translation has been first discovered for $(CAG)_n$ expansion disorders (Zu *et al*, 2011) and was later reported for several other repeat expansions diseases (Cleary & Ranum, 2017). The mechanism remains elusive, but seems to depend on the secondary structure of the repeat RNA. Therefore, several groups have analyzed the RNA structure of $(GGGGCC)_n$ repeats *in vitro* and *in vivo* and found that the repeat RNA can form both so-called G-quadruplexes (G-Qs) and hairpins. G-Qs are four-stranded structures containing stacks of four guanines that associate through Hoogsteen hydrogen bonding within one plane (Fig 1). This structure can form from a single or up to four separate RNA or DNA strands. In contrast to G-Qs formed from DNA, RNA-based G-Qs are more stable and compact as a consequence of more intramolecular interactions. Moreover, RNA G-Qs preferentially assemble in a parallel conformation. Thus, DNA and RNA G-Qs can be selectively targeted. Endogenous RNA G-Qs within untranslated regions and introns regulate transcription, alternative splicing, and protein binding. Hairpins composed of a base-paired stem and a loop are the most common RNA structures and can also affect transcription and alternative splicing. Thus, targeting the secondary structure of the disease-associated $(GGGGCC)_n$ RNA is a potential therapeutic strategy.

In 2014, Su *et al* repurposed a compound originally identified as an interactor of $(CGG)_n$ in fragile X-associated tremor ataxia syndrome for the *C9orf72* repeat RNA (Su *et al*, 2014). This study describes in total three compounds (*1a, 2, 3*), which interfere mainly with the hairpin structure of the $(GGGGCC)_n$ RNA resulting in translational inhibition. In patient-derived neurons, compound *1a* significantly reduced RNA foci and DPR proteins and showed no overt toxicity, but their more potent compound *2*, an aromatic diamidine, was too toxic to validate in patient-derived neurons. None of the compounds were tested in animals.

In contrast, Simone *et al* (2018) specifically targeted the G-Q structure and screened a library of 138 small molecules with known or suspected binding to G-Q structures (Fig 1). They used a FRET-based melting assay for the initial screen and selected three compounds that had a much larger effect on the G-Q formation of $(GGGGCC)_n$ RNA than of $(GGGGCC)_n$ DNA. The three best compounds have a nearly identical atomic structure and are, like the most potent compound in Su *et al*, aromatic diamidines. Circular dichroism spectroscopy confirms direct binding to repeat RNA with 200–400 nM affinity. At 1 μM concentration, two of the compounds reduced RNA foci in iPSC-derived spinal motor neurons and cortical neurons by about 50%. At higher concentration (16 μM) and later time points, both compounds also reduced poly-GP expression by up to 50%, which is likely due to the long half-life time of poly-GP. In contrast to the aromatic diamidine compound from Su *et al*, no toxicity was observed at the effective concentration. In addition to these biochemical and cellular assays, Simone *et al* (2018) fed their best compound (DB1273) to flies modeling $(GGGGCC)_n$ repeat toxicity (Mizielinska *et al*, 2014). Adult flies expressing $(GGGGCC)_{36}$ showed a pronounced reduction in poly-GP. Moreover, feeding larvae with the compound led
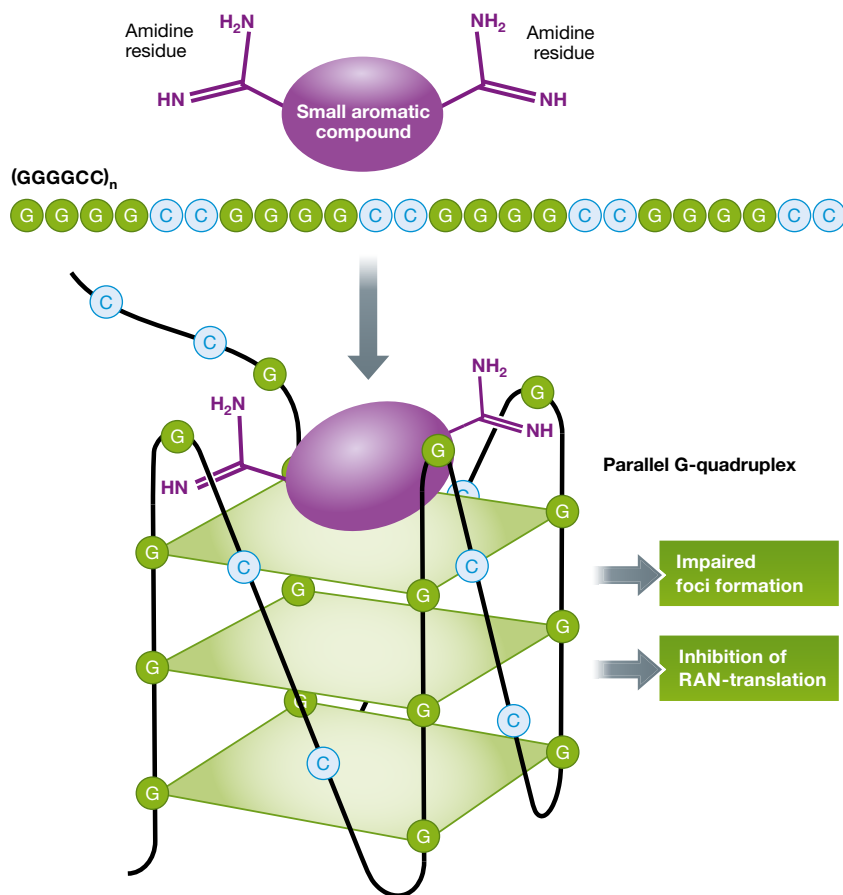

Figure 1. Small molecules stabilize the GGGGCC RNA G-quadruplex structure.

Small aromatic compounds with two amidine residues preferentially interact with the parallel G-Q of the (GGGGCC)$_n$ repeat RNA and stabilize this structure. Stabilization of the G-quadruplex structure reduces RNA foci formation and inhibits repeat translation. Note that other G-Q confirmations are possible.

to a modest increase in survival. Since poly-GR is the main driver of toxicity in this model, Simone *et al* (2018) also developed a new poly-GR immunoassay to directly show effect on the main toxic species. Indeed, stabilizing the G-Q structure of (GGGGCC)$_{36}$ using DB1273 also reduced poly-GR levels by 33%, which is consistent with the moderate survival benefit. Since brain penetrance of DB1273 is still low, medicinal chemists may be able to improve the *in vivo* effects significantly in the future.

The Isaacs laboratory is covering a lot of ground already by validating their novel compounds in a fly model. The next obvious step would be treating mice expressing the (GGGGCC)$_n$ repeat. One strategy to improve delivery to the CNS might be biopharmaceutical modifications such as incorporation into nanospheres or nanocapsules (Lu *et al*, 2014). Another way to boost the efficiency of the compounds could be *in situ* CLICK chemistry,

because 1,3-dipolar cycloaddition of alkynes and azides could promote cross-linking of the modified compounds to (GGGGCC)$_n$ G-Qs *in vivo* (Di Antonio *et al*, 2012). The report from Simone *et al* (2018) also highlights a gradual shift in the drug discovery world. In 2017, targeting specific RNA molecules by small molecules is becoming increasingly feasible (Bernat & Disney, 2015), although antisense oligonucleotides still have superior efficacy. However, antisense oligonucleotides require invasive application and come with a hefty price tag (Jiang *et al*, 2016).

What do these new findings mean for our understanding of the pathological hallmarks of *C9orf72* disease? Apparently, stabilizing either the G-Q or the hairpin structure can reduce RNA foci formation and inhibit translation of the (GGGGCC)$_n$ repeat. However, it is unclear whether the compounds actually dissolve RNA foci or just impair their detection. It also remains elusive whether these

compounds specifically inhibit RAN translation or would also inhibit ATG-initiated translation of the structured repetitive RNA. RAN translation seems to highly depend on the RNA structure, because only (CAG)$_n$ but not (CAA)$_n$ repeats are translated into poly-Q in the absence of an ATG-start codon (Zu *et al*, 2011), suggesting that targeting the secondary structure of RAN-translated repeat RNAs is a potential strategy to slow or stop disease progression. A compound affecting RAN translation of different repeat RNAs might be beneficial for many diseases (Cleary & Ranum, 2017).

In conclusion, this paper is an encouraging and timely study, because our understanding of *C9orf72* pathogenesis is growing rapidly and the first treatment options, such as antisense oligonucleotides are already on the horizon (Jiang *et al*, 2016). Patients can only benefit from the intense interest in the *C9orf72* mutation as academia and industry race for a treatment.

Acknowledgements

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